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Relevance of germline mutation screening in both familial and sporadic head and neck paraganglioma for early diagnosis and clinical management

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Abstract. *Background*: Head and neck paraganglioma (PGL) are benign tumors that can cause important direct or surgery induced morbidity. Almost all familial and 11–29% of sporadic PGL are caused by inactivating germline mutations in succinate dehydrogenase (SDH) genes. Our aim was to screen for such mutations and to evaluate clinical parameters as predictors of germline mutation.

Methods: Seventy-four PGL patients were analyzed for germline mutations and large deletions in SDH genes, VHL and RET. Results were correlated to clinical characteristics including gender, age, tumor localization and multifocality. The surgical approach was evaluated in terms of tumor origin, sequelae and subsequent evolution.

Results: Mutations in SDHB and SDHD were identified in equal proportion in 13/13 (100%) of familial and in 15/61 (25%) of sporadic cases. Familiarity, age $\leqslant$ 50 years and male gender were predictors of any germline mutation, while multifocality and carotid/vagal localization were indicative of SDHD mutation in particular.

Conclusion: In contrast to other series, this cohort of Spanish patients showed many SDHB mutations. Sporadic cases with germline mutation are frequent and underline the importance of mutational screening of all PGL patients, allowing the identification of relatives at risk and the early diagnosis of the disease, reducing or avoiding morbidity.

Keywords: Paraganglioma, SDH, mutation, large deletion, genetic screening

1. Introduction

Paragangliomas (PGL) are uncommon tumors. They represent 0.012% of all cancer and 0.6% of head and neck tumors, mostly carotid body followed by jugular, tympanic, and vagal paraganglia. Other, less frequent locations are: larynx, nasal cavity, orbit and trachea [24]. Head and neck paraganglia are associated with the parasympathetic nervous system and are found near arteries and cranial nerves of the bronchial arches, with a cervicocephalic distribution [24]. Clinical manifestations of PGL depend on the site of the tumor [9]. Carotid PGL classically appear as slow-growing asymptomatic cervical masses, rarely causing late symptoms as a result of cranial nerve invasion. Vagal tumors present as a parapharyngeal mass frequently affecting the lower cranial nerves. Jugular and tympanic tumors are accompanied by pulsatile tinnitus, hearing loss and other lower cranial nerve deficits, depending on their location and size [9,24]. A 10–15% of tumors demonstrate aggressive behavior, with relatively fast progression. Jugular and vagal paragan-

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gliomas are generally diagnosed in an advanced stage, with a third of the patients showing different degrees of intracranial invasion [30]. This implies a lower cranial nerve dysfunction, which includes dysphagia, hoarseness, aspiration, tongue paralysis, shoulder drop and voice weakness.

PGL are considered benign tumors although they sometimes have a biological behavior similar to malignant tumors. Malignant PGL is defined by the presence of nodal or distant metastasis. Familial forms and tumors found in the proximity of the organ of Zuckerkandl have the greatest malignant potential (14–50%) [5]. The prevalence of malignant PGL depends on the site: 1.41% for carotid body PGL, 5.1% for jugulo-tympanic and 10% for vagal PGL [26]. The most common sites for remote metastasis are the lung, bone, breast, pancreas, thyroid, kidney and liver [30]. Multi-centric or bilateral tumors may occur in 10% of cases. In the case of familial tumors, 30–40% starts out as either bilateral PGL, or multi-centric PGL in different areas of the cervicofacial region or in the sympathoadrenal paraganglionic tissue of the abdomen [5,24]. An estimated 1–3% of PGL are functioning and present symptoms related to noradrenaline secretion [24].

PGL can be sporadic or familial. Familial PGL, with a prevalence between 10% and 50% [2,34], form a genetically heterogeneous entity, associated with germline mutations in succinate dehydrogenase genes SDHB, SDHC and SDHD, and occasionally in VHL and RET [2,5]. Mutations in SDH genes are the cause of nearly all of familial cases, while approximately a quarter of all apparently sporadic PGL also present germinal alterations in SDH, being in fact "occult familial" cases. In the remaining sporadic cases, the triggering molecular alteration is unknown [5]. Studies on PGL tumors aiming to find inactivating somatic mutation or methylation concerning SDH genes have given negative results [8,10,14], although somatic SDHB and SDHD mutations have been reported in pheochromocytoma [12,33].

The aim of this study is to analyze clinical parameters as predictors of germline mutations. Early detection of a familial paraganglioma will allow early surgical treatment and, consequently, lead to reduced morbidity.

2. Material and methods

2.1. Patients

This study included 74 PGL patients diagnosed with head and neck PGL between 1981 and 2007 in the Hospital Universitario Central de Asturias in Oviedo, Spain. Clinico-pathological information and family history were obtained by direct interviews and review of the medical charts. Cases were considered to be familial when at least two first-degree relatives, preferably from different generations, or two second-degree relatives were affected by PGL.

Our study group consisted of 51 female and 23 male patients, with 22 carotid body, 29 jugular, 14 tympanic and 9 vagal paragangliomas. Thirteen patients had PGL or PCC carrying relatives. The median age at the time of diagnosis was 50 years (range 16–79). A detailed overview of all clinical features is given in Table 1. Informed consent was obtained from all patients and the study was approved by the ethical committee of our institute. The follow-up time was 1–19 years (a mean of 8 years) and included clinical examination as well as serial MRI scans.

2.2. Mutation analysis

Blood samples have been collected in EDTA tubes. Genomic DNA was extracted from peripheral blood leucocytes using the Qiagen Blood Kit (Qiagen GmbH, Hilden, Germany). All patients were studied for germline mutations in all exons and splice sites of SDHB, SDHC, SDHD and VHL and in exons 10, 11, 13 and 16 of RET by direct sequencing using

Table 1
Clinical features of 74 PGL patients

	Total	Carotid	Jugular	Tympanic	Vagal
	74	22	29	14	9
Female	51	14	19	12	6
Male	23	8	10	2	3
Age $\leqslant$ 50 year	38	11	16	5	6
Age $>$ 50 year	36	11	13	9	3
Sporadic	61	16	25	14	6
Familial	13	6	4	0	3
Unique PGL	67	19	28	14	6
Multiple PGL	7	3	1	0	3
Without PCC	72	21	28	14	9
With PCC	2	1	1	0	0
Non-functioning PGL	70	20	28	14	8
Functioning PGL	4	2	1	0	1
Benign	72	22	27	14	9
Malignant	2	0	2	0	0
No recurrence	68	22	23	14	9
Recurrence	6	0	6	0	0

the ABI PRISM 3100 and 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sense and antisense sequencing was performed for confirmation. Primers and conditions are given in Table 2. In addition, large deletions were analysed by multiplex ligation-dependent probe amplification (MLPA) for VHL (Salsa P016B), and for SDHB, SDHC and SDHD (Salsa P226) (MRC-Holland, Amsterdam, The Netherlands), according to the manufacturer's recommendations.

Mutations were annotated according to the official nomenclature from the Human Genome Variation Society (http://www.hgvs.org) using the following reference sequences: SDHD (NT_033899.7) and SDHB (NT_004610.18). The variants were classified into four groups (pathogenic, UV-III, UV-II and UV-I) based on the literature and on in silico analyses using the following online software: http://blocks.fhcrc.org/sift/SIFT.html (evolutionary conservation), Granthamscore [13] and http://www.russell.embl.de/aas/ (amino acid properties), http://www.fruitfly.org/seq_tools/splice.html, http://www.cbs.dtu.dk/services/NetGene2/ and http://violin.genet.sickkids.on.ca/∼ali/splicesitefinder.html (splicing effects). An unclassified variant (UV) is a variant with unknown pathogenicity; UV-III means probably pathogenic, UV-II is of unclear pathogenicity and UV-I is a probably not pathogenic variant. The mutations described previously in the literature can also be found in the SDH database: http://chromium.liacs.nl/lovd_sdh [3].

2.3. Statistical analysis

The statistical software package SPSS for Windows v.12 (SPSS® Inc., IL, USA), was used to perform the analyses. Fisher Exact, Pearson Chi-square test for nominal variance was used for correlation. For clinical predictors of mutation, the relative risks were calculated.

Table 2
PCR primers used in the mutational analysis

Gene	Exon	Primer F	Primer R
VHL	1	tccggcccgggtggtctggat	tgctatcgtgccagacttcgg
	2	attacaggtgtgggccac	aacggactacagtccgtgcc
	3	gttccttgtactgagaccta	aatgtgacaaagtagagtcg
RET	10	aggaggctgagtgggctacg	gtgtagactccagggttgttcc
	11	tacccagtggtgcccagcct	tcgacggaaggtccctccct
	13	cttccaggagcgatcgtttg	gtcggtgacgtgggtccgac
	16	tccttcctagagagttagag	cctccaatgtctttgtggga
SDHB	1	tgtaaaacgacggccagtgccgctactgcgctattg	caggaaacagctatgaccgctttcctgacttttccc
	2	tgtaaaacgacggccagttctgttgtgccagcaaaatg	caggaaacagctatgaccgccttccaaggatgtgaaaa
	3	tgtaaaacgacggccagtacatccaggtgtctccgatt	caggaaacagctatgacccaagcctctttggaagacca
	4	tgtaaaacgacggccagtatttggggcaggactgattc	caggaaacagctatgacccaaatcctgccctgaaaaac
	5	tgtaaaacgacggccagtaagctgaggtgatgatggaa	caggaaacagctatgacccacactcctggcaatcatctt
	6	tgtaaaacgacggccagtcaagtaggcactttgttcatgc	caggaaacagctatgacctggctggcttacagcaatct
	7	tgtaaaacgacggccagtcccagagctttgagttgagc	caggaaacagctatgacctagggttgctctctgccaat
	8	tgtaaaacgacggccagtgggttttccctttcagtttca	caggaaacagctatgacctgctgtattcatggaaaaccaa
SDHC	1	tgtaaaacgacggccagtgtcacatgacacccccaac	caggaaacagctatgacccccaggcacaggataaacag
	2	tgtaaaacgacggccagttctatcccttcacccctaaaaa	caggaaacagctatgaccagcgagactccgtctcaaaa
	3	tgtaaaacgacggccagtaaacgttatgcaaaatattaaaccaa	caggaaacagctatgacccccttcagaactttcacccact
	4	tgtaaaacgacggccagtgccaagatagactctctactatggtg	caggaaacagctatgaccttcaaaggaggcggagacta
	5	tgtaaaacgacggccagtcaggggtcccagttttatgt	caggaaacagctatgaccagtctccccactcccttcac
	6A	tgtaaaacgacggccagtttttttgctttgtccacagatg	caggaaacagctatgaccaaggagaacttttcccaggc
	6B	tgtaaaacgacggccagtaaggtggggcataagggtag	caggaaacagctatgaccgccccatagaggacaacaca
SDHD	1	tgtaaaacgacggccagtgttcacccagcatttcctctt	caggaaacagctatgacctgctgtgatttcggtattttc
	2	tgtaaaacgacggccagtatgttatcccctatttattgtt	caggaaacagctatgacctctgcccaaaggtgtaaacta
	3	tgtaaaacgacggccagtcattgagataccttgttgtgctaa	caggaaacagctatgaccttcaatcaacttctccctcata
	4	tgtaaaacgacggccagtgtggagtggcaaatggagaca	caggaaacagctatgacctctgttatttcttccttattgtga

3. Results

3.1. Clinical

A cervical approach was performed in all 22 patients with carotid PGL. Only one patient presented preoperatively with a vagal nerve paralysis, and 4 patients more showed postoperatively different combinations of lower cranial nerve paralysis. In all patients the tumor was completely excised with no recurrences during follow-up. Two of the three patients with bilateral carotid tumors developed blood pressure control disturbances after resection of the tumors. In vagal PGL the most commonly used approach was exclusively cervical (7 patients), although two more patients required an additional transpetrosal exposure of the jugular fossa; postoperative facial nerve paresis was observed in these latter two patients. Cranial nerve X was affected prior to surgery in $4/9$ (44%) patients with vagal PGL. A new paralysis appeared during the postoperative period in $4/9$ (44%) additional patients. Only one patient experienced a recurrence in the follow-up period.

In patients with large jugular PGL, an exposure of the jugular fossa with complete mobilization of the intrapetrous facial nerve was used. Smaller tumors could be managed without mobilization of the facial nerve or with a short mobilization. The most frequent postoperative, neurological complications were facial and lower cranial nerve paralysis. The facial nerve was found to be clinically affected prior to surgery in 2 patients with multiple PGL. As a result of the surgery, some degree of facial paresis was observed in $23/29$ (79%) patients. The immediate post-operative facial function in jugular PGL treated by complete transposition of the facial nerve was normal to slight paresis in only 24% of patients. In the remaining worst affected cases, an improvement in nerve function was achieved to the point of attaining a grade III one year after the operation. However, in patients treated with less than a complete facial nerve transposition due to the smaller size of the tumor, post-operative facial function was normal to slight paresis in 77% of cases. Lower cranial nerves were affected prior to surgery in $10/29$ (34%) patients with jugular PGL. Apart from these patients, new paralysis appeared during the post-operative period in $9/29$ (31%) patients undergoing surgery. Pre and postoperative damage to the vagus nerve correlated with the size of the tumor and intracranial involvement. Cranial nerve paralysis rate was correlated with the size of the tumor. Tympanic, due to its small size had no cranial nerve complications. Of 52 recorded cases, 23 tumors had a maximum diameter less than 3 cm and 29 tumors larger than 3 cm. Only one (4%) patient with a tumor < 3 cm vs. 14 (48%) patients with a tumor > 3 cm showed cranial nerve paralysis preoperatively (Fisher Exact Chi^2 $p = 0.001$). After the operation, 4 (17%) patients with a tumor < 3 cm vs. 20 (69%) patients with a tumor > 3 cm presented with a new paralysis postoperatively (Fisher Exact Chi^2 $p = 0.000$). Six patients (20.7%) recurred postoperatively: in two patients, the recurrence was small and controlled by surgery; more extensive recurrences were treated in 4 cases by radiotherapy only.

Finally, with the exception of a patient with multiple PGL, all tympanic PGL were of small size and therefore resected through otological approaches. This means that patients recovered the hearing loss with disappearance of the pulsatile tinnitus, and they had no significant sequelae.

3.2. Genetic

A total of 28 cases carried germline mutations, 14 in SDHB and 14 in SDHD. No mutations or large deletions were found in SDHC, VHL or RET. All mutations in SDHB and SDHD were distributed over all exons with no apparent hotspot. Of all 28 mutations, 13 were missense, 12 frameshift, two splice site, and MLPA analysis revealed one case with a deletion of exon 3 of SDHB. Missense mutations were predominantly observed in SDHB ($10/14$) and frameshift mutations were most frequent in SDHD ($10/14$). Table 3 provides an overview of all mutations detected.

Five mutations have not previously been described in literature, according to the SDH database (http://chromium.liacs.nl/lovd_sdh/) [3]. Based on in silico analyses (Table 3) three of them were classified as pathogenic: SDHB exon 3 deletion c.201−?_286+?del (EX3del), SDHD exon 2 splice site mutation c.53-2A $>$ G which may lead to skipping of exon 2, and SDHD exon 3 frameshift mutation c.234_243del TCTGCTTCCG [p.Pro81IlefsX2]. The fourth, SDHD exon 3 missense mutation c.230T $>$ G [p.Leu77Arg], sits in a highly conserved region of the gene and was considered as probably pathogenic (UV-III). Finally, SDHD exon 4 missense mutation c.367G $>$ A [p.Ala123Thr] was considered as UV-II, a variant of uncertain pathogenicity because it sits in the functional domain but the amino acid change is not very marked. The other 23 mutations detected have been published previously (Table 3). In total 18 mutations were considered pathogenic, 6 probably pathogenic (UV-III) and 4 of unknown pathogenicity (UV-II).

Table 3
Description of all identified germline mutations

	Loc	Sex	Age	Fam	Gene	Exon	Mutation	Predicted effect	Type	Path.
1	jug	F	61	No	SDHB	2	c.166_170delCCTCA	p.Pro56TyrfsX5	Frameshift	Path.
2*	vag	M	42	Yes	SDHB	3	c.201−?_286+?del (EX3del)	?	Exon del	Path.
3	jug	M	31	No	SDHB	3	c.269G > A	p.Arg90Gln	Missense	UV-II
4	jug	F	32	Yes	SDHB	3	c.277T > C	p.Cys93Arg	Missense	UV-III
5	jug	M	39	Yes	SDHB	4	c.293G > A	p.Cys98Tyr	Missense	UV-III
6	jug	M	16	Yes	SDHB	4	c.312insCACTGCA	p.Ile105HisfsX16	Frameshift	Path.
7	jug	M	39	No	SDHB	5	c.540G > A	p.Leu180Leu	Splice site	Path.
8	vag	F	29	Yes	SDHB	6	c.557G > A	p.Cys186Tyr	Missense	UV-III
9	car	M	70	Yes	SDHB	6	c.589C > T	p.Pro197Ser	Missense	UV-III
10	jug	F	47	No	SDHB	6	c.589C > T	p.Pro197Ser	Missense	UV-III
11	jug	M	43	No	SDHB	7	c.688C > T	p.Arg230Cys	Missense	Path.
12	jug	M	50	No	SDHB	7	c.689G > A	p.Arg230His	Missense	UV-II
13	car	F	54	No	SDHB	7	c.725G > A	p.Arg242His	Missense	UV-II
14	car	F	45	Yes	SDHB	7	c.761C > T	p.Pro254Leu	Missense	UV-III
15	car	F	45	Yes	SDHD	1	c.14G > A	p.Trp5X	Frameshift	Path.
16	car	F	25	No	SDHD	1	c.33C > A	p.Cys11X	Frameshift	Path.
17	car	M	43	Yes	SDHD	1	c.50G > T	p.Arg17Leu	Missense	UV-II
18*	car	M	48	No	SDHD	IVS1	c.53-2A > G	Skipping of exon 2	Splice site	Path.
19	car	F	53	No	SDHD	2	c.106C > T	p.Gln36X	Frameshift	Path.
20	jug	F	46	Yes	SDHD	2	c.120_127delCCCAGAAT	p.Ile40MetfsX26	Frameshift	Path.
21	car	M	38	No	SDHD	2	c.129G > A	p.Trp43X	Frameshift	Path.
22*	jug	F	46	No	SDHD	3	c.230T > G	p.Leu77Arg	Missense	UV-III
23*	vag	F	60	No	SDHD	3	c.234_243delTCTGCTTCCG	p.Pro81IlefsX2	Frameshift	Path.
24	vag	F	24	No	SDHD	4	c.337_340delGACT	p.Asp113MetfsX21	Frameshift	Path.
25	car	M	44	Yes	SDHD	4	c.337_340delGACT	p.Asp113MetfsX21	Frameshift	Path.
26	vag	M	40	Yes	SDHD	4	c.337_340delGACT	p.Asp113MetfsX21	Frameshift	Path.
27*	jug	F	50	No	SDHD	4	c.367G > A	p.Ala123Thr	Missense	UV-II
28	car	F	22	Yes	SDHD	4	c.386dupT	p.Leu129fs	Frameshift	UV-III

Notes: Loc – localization; vag – vagal; car – carotid body; jug – jugular; Fam – familial; Path – pathogenic; UV-II – unclassified variant with unknown pathogenicity; UV-III – unclassified variant probably pathogenic. Classifications were based on "in silico" analyses as described in Section 2. All mutations except those marked with an astrisk (*) have been described before.

3.3. Clinico-genetic correlations

After controlling for all other variables in the logistic regression model, the relative risks and p-value of each independent variable were calculated. Familiarity was strongly predictive for the presence of germline mutation with a relative risk of 4.1 ($p = 0.000$). All 13 familial PGL carried germline mutations both in SDHB as in SDHD with a distribution (7 and 6, respectively) not different to the 15 mutation positive sporadic cases (7 and 8, respectively). Other clinical factors predictive of germline mutations were age < 50 (relative risk 4.36; $p = 0.025$), multiple HNPGL relative risk 3.19; $p = 0.001$), male gender (relative risk 1.92; $p = 0.025$) and carotid/vagal localization (relative risk 1.93; $p = 0.027$). Factors as previous PCC, malignancy, functionality or recurrence did not reach significance, due to the low number of cases with these characteristics.

Factors that predicted germline of SDHB or SDHD were multiple HNPGL, tumor localization and to a lesser extend sex. All 7 cases with multiple HNPGL carried mutation in SDHD. PGL of the carotid body had mostly mutations in SDHD (8 of 11), whereas jugular PGL showed mostly SDHB mutations (9 of 12) (Table 4). Neither of the 14 tympanic PGL harbored a germline mutation, but one patient with a primary vagal PGL, carrier of a SDHD mutation, had multiple PGLs including one tympanic.

Recurrences were not related to any of the clinical features, except for the tumor localization: only jugular and vagal PGL developed recurrences, due to incom-

Table 4
Clinical features related to germline SDH mutations

Clinical parameters	Variables	Mut pos (%)	SDHB	SDHD
Localization	22 carotid	11 (50%)	3 (14%)	8 (36%)
	29 jugular	12 (41%)	9 (31%)	3 (10%)
	14 tympanic	0 (0%)	0 (0%)	0 (0%)
	9 vagal	5 (55%)	2 (22%)	3 (33%)
Sex	51 female	15 (29%)	6 (12%)	9 (18%)
	23 male	13 (57%)	8 (35%)	5 (22%)
Age $\leqslant$ 50	38 yes	23 (61%)	11 (29%)	12 (32%)
	36 no	5 (14%)	3 (8%)	2 (6%)
Familial history of PGL	61 no	15 (25%)	7 (11%)	8 (13%)
	13 yes	13 (100%)	7 (54%)	6 (46%)
Multiple PGL	67 no	21 (31%)	14 (21%)	7 (10%)
	7 yes	7 (100%)	0 (0%)	7 (100%)
Total number	74	28 (38%)	14 (19%)	14 (19%)

plete resection in large intracranial involvement, irregular growth pattern of the tumor, and surgical difficulties in a deep, complex and extremely vascularized region. A full description of the mutations in relation to clinical parameters is given in Table 4.

4. Discussion

4.1. Clinical aspects

Our series of 74 cases paralleled those described in other series with regard to the distribution of sex, age and tumor localization, although jugular and tympanic were somewhat more frequent than carotid and vagal PGL (respectively 58% and 42%), probably due to the fact that our department is a center of reference of skull base tumors in Spain. Thirteen of 74 (17%) patients had relatives with PGL or PCC, which is within the range of 11–32% in other published series. Finally, we had slightly fewer patients with malignant PGL (3%) [17], with previous PCC (3%) or with multiple PGL (9%) [9,24].

Total removal of jugular and vagal PGL very often leads to lower cranial nerves paralysis, severe or total hearing loss, and temporary facial palsy. In a published series of 152 jugular paragangliomas, complete removal of the tumor was carried out with 69% of lower cranial nerves palsy [15]. Similar rates of pre and postoperative vagal palsy were observed in our series (65%). Improvement of surgical techniques has reduced the incidence of postoperative facial palsy. According to Sanna et al., the surgical approach without facial nerve transposition achieves excellent results with 72% of normal facial nerve function in the long term [27]. Our results are comparable, with an incidence of 77% of normal to slight paresis postoperative facial nerve function.

There is currently a trend to restrict surgical treatment of PGL, as this is a disease that does not generally result in death, while surgery can cause important sequelae [31]. Most carotid PGL are treated surgically as complications are uncommon. Generally, carotid PGLs do not infiltrate the carotid artery even in the case of encasement of the artery. Resection of vagal PGL almost always entails vagal paralysis, and therefore several authors question the need for surgery, unless the tumor has already produced lower nerve paralysis or in the case of young patients [21]. Tympanic PGL usually are of small size and are easily removed by a tympanomastoid approach with rare morbidity; therefore they must be operated on to prevent loss of hearing and pulsatile tinnitus. There is some controversy regarding the therapeutic strategies in jugular PGL. A wait and see policy using interval scanning is a valid option in many jugular PGL, particularly in patients over 50 years of age and normal lower cranial nerves function. In the case of tumor growth external-beam radiotherapy or stereotactic radiation therapy have been advocated, with a local control rate (no significant growth of the tumor) ranking from 73–93% of patients [19].

4.2. Frequency of mutations

Although mutations in the PGLs of head and neck are reported more prevalent in SDHD than in SDHB

[1,2,4,7,11,20,23,29] in our study both mutations were observed in equal proportion: 14 cases with mutation in the SDHB and 14 cases in the SDHD. Inactivating germline mutations in SDHB and SDHD were involved in 100% of familial cases and 25% of non-familial cases, similar to other published series [1,2,4, 7,11,20,23,29]. Notably, in the familial cases, SDHB concerned 54% of the mutations while in all earlier described head and neck PGL series SDHD is with distance the most frequent gene mutated. But also the 11.5% of SDHB mutations in sporadic (i.e. occult familial) cases were more frequent than reported previously, with the exception of a study of a Belgian cohort of 30 sporadic cases that identified 8 (27%) SDHB mutations [25].

Mutations in SDHB and SDHD were distributed in all the exons, with the exception of exons 1 and 8 of SDHB, whereas those of SDHD were distributed in all the 4 exons, but most frequently in exons 4 and 2. The predominance of missense over truncating mutations in SDHB and vice versa in SDHD has been described before but was even more pronounced in our series. The difference may be explained by the fact that SDHD has a lower degree of conservation than SDHB, and would need more deleterious mutational changes to have a pathogenic effect [3].

Most of the mutations identified in this study have been described previously in the literature, however, the pathogenicity of some of them remains to be proven. Four of six mutations that we annotated as probably pathogenic (UV-III, cases 4, 8, 9 and 28) were found in familiar PGL cases, and moreover, sporadic case n°10 carried the same UV-III mutation as familiar case 9. This makes it very likely that these five UV-III mutations are in fact pathogenic. We found five new mutations, based on in silico analyses three were considered pathogenic, one as probably pathogenic (UV-IIII) and one of unknown pathogenicity (UV-II). We found three patients with the same p.Asp113MetfsX21 mutation in SDHD exon 4, and two patients had the same p.Pro197Ser mutation in SDHB exon 6. However, as far as we could check the possibility of a familial relation between these patients is very unlikely.

In our study, mutations or deletions in exons of SDHC, VHL or RET genes were absent in sporadic and familial cases, in agreement with other studies [1, 2,25], although SDHC mutations have been reported in approximately 4% of cases [5].

4.3. Clinical predictors of germline mutation

As reported earlier, carotid and vagal PGLs were more common among familial cases (9/31 vs. 4/43, Chi^2 $p = 0.030$) and carried more frequent mutations compared to jugular/tympanic tumors (16/31 vs. 12/43, Chi^2 $p = 0.034$) [2]. Furthermore, our data confirmed the finding that carotid and vagal PGLs have more mutations in SDHD, while jugular PGLs mostly carry mutations in SDHB (Chi^2 $p = 0.027$) [2]. Tympanic PGL deserve special mention. All 14 tympanic PGLs in our series were of small size, occurring preferentially in women (12/14) with a mean age of 55 years. Curiously, we found no mutations in tympanic PGL patients. There was one exception of a patient with multiple PGLs, where an extended tympanic PGL was associated to vagal and carotid body PGLs. This patient carried a SDHD mutation. Specific reference to tympanic PGL is scarce in the literature. In a German cohort, Schiavi et al. reported 27 tympanic cases among which there were 2 carrying a SDHC and 4 SDHD mutation [29]. Hereditary paragangliomas present earlier than sporadic cases (mean 39 years vs. 52 years), with a trend for earlier age of tumor onset with successive generations. In our series, an age limit of 50 years predicted the presence of germline mutation better than 40 years [18,23]. Sixty-one percent of patients of 50 years or younger vs. 16% of patients over 50 years carried a mutation (Chi^2 $p = 0.000$), while 61% of patients of 40 or younger vs. 30% of patients over 40 had a mutation. Patients with either SDHB or SDHD did not differ in age. Male patients had more frequent mutations than females (57% vs. 29%, Chi^2 $p = 0.025$), and in particular more in SDHB (8/13 vs. female 6/15 Chi^2 $p = 0.225$). This is as described earlier.

Although most of the PGLs present as a single mass, multiple PGLs are seen in approximately 10% of cases [24]. Our study population showed comparable results to those described in the literature, with a frequency of 9% of multiple tumors [24]. Multiple PGLs are generally confined to the head and neck region but, occasionally, they may be accompanied by paragangliomas in the abdomen. Nevertheless, in sporadic cases the incidence of multiple tumors probably represents unrecognized familial cases, because the chance of developing multiple PGLs without a predisposing factor is small [32]. Multiple PGLs are almost exclusively related to mutation in SDHD. All seven cases (patients 16, 20, 23–26 and 28, Table 4) in our series were SDHD mutation carriers (Chi^2 $p = 0.003$). Four of the seven patients had family members with PGL, and in two cases (n° 25 and 28), these family members also had multiple PGL. There was an additional patient (n° 15) with a single carotid tumor who had two relatives with mul-

tiple PGLs. Also this patient carried a SDHD mutation. Curiously, three of the multiple PGL patients carried the same Asp113fs(X21) mutation, but we could not find any specific literature concerning this mutation that may explain the relevance of this finding.

Malignant PGL occurs with a frequency that ranges between 6–9%, a number superior to that found in our series, where only 2 cases were observed (3%). A cause-specific survival rate of 94% has been reported in benign jugular paragangliomas and of 89% in vagal paragangliomas [19], whereas the 5-year survival rate of the uncommon malignant paragangliomas is 60% [16]. Malignant tumors have been associated to SDHB mutation and has been observed more frequently in vagal PGL and in secreting tumors [5,17]. In our series one case presented with intracranial extension and neck node metastasis and another with cerebral metastases. However, both our cases were sporadic cases, jugular and non-secreting PGL. During the follow-up six patients with jugular PGLs presented a tumor recurrence. There was a correlation between the recurrence and the size and location of the tumor, with no influence of the existence of mutations in SDH genes. One case had a mutation in SDHB, one in SDHD and four did not have a germline mutation.

4.4. Genetic screening and clinical management

Cervical MRI has been advocated as the initial screening tool for familial single or multicentric PGLs due to mutation of SDHD, because most of them are located in the head and neck area. On the other hand, 6-[(18)F]fluorodopamine positron emission tomography [(18)F-DOPA PET] has the advantage on the cervical MRI of a higher accuracy in PGLs smaller than 1 cm and to allow screening of the whole body, what is of particular interest in PGLs due to mutations in SDHB. With the same purposes, radiolabelled compounds that bind to receptors, as the Pentetreotide, have also been successfully used in the screening of familial PGLs [9, 24,34].

Although germline mutations in SDHD have been described in the literature as predisposing for head and neck paragangliomas, our cohort of 74 Spanish patients, both familial and sporadic, showed almost equal frequencies for SDHB and SDHD. No patients carried mutations in SDHC, VHL or RET. Clinical predictors of germline mutations were: having family members with PGL or PCC, having multifocal PGL and age younger than 50 years. SDHD mutation was seen exclusively in multifocal PGL and significantly more frequent in carotid and vagal PGL. SDHB mutations were more frequent in jugular PGL, and tympanic PGL did not carry mutations.

These correlations are important in guiding the genetic testing of new PGL patients; for some patients it may be better to start with SDHD, and for others with SDHB, which is also relevant from an economical point of view, as indicated by Neumann et al. [22]. On the other hand, it may be recommendable to screen for mutations in all PGL patients, irrespective of clinical indicators of germline mutations. Given the low incidence of these patients and the relatively low workload of the genetic screening, the costs of this approach should not be too high considering the benefits. Hospitals where genetic screening is technically or economically difficult, should seek collaboration with bigger clinical centers or with research groups in order to obtain the best results for their patients and to advance the research on PGL. The identification of germline mutations is of paramount importance because it gives opportunity for early diagnosis through periodical image studies in family members of the affected patient. Early detection of a familial paraganglioma allows early surgical treatment, that even in the jugular fossa can at this stage be carried out without any damage to hearing, facial nerve and lower cranial nerves, thus reducing the complication rate because that correlates strongly with the size of the tumor.

Taking into account that approximately 25% of all apparently sporadic PGL also present germinal alterations, a percentage perhaps even higher when additional disease-related genes are discovered, genotypic analysis as a screening tool in family members of all affected patients should play a front-line diagnostic role, leading to more timely and cost-effective patient management, reducing or avoiding morbidity.

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